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REVIEW

Application of quantitative ^{31}P NMR in biomass lignin and biofuel precursors characterization

Yunqiao Pu,[ac] Shilin Cao[b] and Arthur J. Ragauskas*[bc]

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The last decade has seen tremendous growth and interest in renewable energy and fuels aimed primarily at addressing issues of climate change, energy security, and rising energy costs. These efforts coupled with the demand for efficient utilization of biomass place a premium on the detailed analysis of the fundamental chemical structures of biomass, especially in light of the ever-increasing efforts to generate transgenic plants with reduced recalcitrance and altered lignin structure. This review examines the growing application of phosphitylation followed by ^{31}P NMR to quantitatively analyze biomass lignin structures including guaiacyl, syringyl, guaiacyl with carbon substituents at the C_5 position, catechol, *p*–hydroxyphenyl, aliphatic and carboxylic hydroxyl groups. The application of this methodology to provide a rapid analytical tool for lignin/biomass derived bio-oils and biodiesel precursors is also discussed. Utilizing lignin isolated from native and transgenic plants as well as from pretreatment and biological/thermal deconstruction processes, researchers have demonstrated that this technique has unique characterization capabilities which have broad applicability in the biofuels research community.

1. Introduction

The need for development of sustainable industrial technologies has become a global theme that is impacting numerous fields including translational research in renewable energy and fuels. Spurred by the need to address issues about climate change, energy security, and ever-increasing demand for limited petroleum resources, governmental support and societal interests have prompted entrepreneurs and researchers to develop new viable approaches to conversion of biomass to biopower and lignocellulosic-based biofuels.[1–6] The utilization of bioresources for alternative fuels production provides a long-term sustainable option for fuels production which can be accomplished in an environmentally compatible manner for many regions of the globe.[7–10] For example, the USDA/DOE Billion Ton report has documented how the biomass reserves in the United States have the potential to address approximately one third of U.S. petroleum demand provided that nontraditional resources of biomass are utilized including energy-crops, under-utilized forest resources, agricultural and forest residues.[11]

[a]*Institute of Paper Science and Technology, Georgia Institute of Technology, Atlanta, GA, USA*
[b]*School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, USA. E-mail: arthur.ragauskas@chemistry.gatech.edu; Fax: (+404) 894-4778; Tel: (+404) 894-9701*
[c]*BioEnergy Science Center, USA; Web: http://bioenergycenter.org*

Broader context

From an overview perspective, the conversion of biomass to biofuels requires the depolymerization of biopolymers such as cellulose, hemicellulose and/or lignin followed by the removal of hydroxyl group functionality to yield less polar more hydrocarbon-like fuels. Recent research studies have shown that phosphitylation of hydroxyl groups followed by quantitative ^{31}P NMR provides a valuable characterization tool for several biofuel technology platforms including pyrolysis oils, biodiesel, and biological conversion of biomass to second and third generation biofuels. Utilizing this methodology and well established databases of chemical shifts it is now possible to routinely determine the content of aliphatic hydroxyl groups, phenolic (*i.e.*, guaiacyl, syringyl, C_5- substituted guaiacyl phenolics, catechols, *p*–hydroxyphenols, *etc.*), carboxylic acid hydroxyl groups and water in assorted biomass related samples with limited NMR access time. Furthermore, one of the most promising technologies to reduce the intrinsic recalcitrance of biomass involves the development of transgenic plants with altered lignin structures and contents. This review summarizes recent advances in the application of the phosphitylation/^{31}P NMR methodology, as it applies to transgenic and native lignin, to illustrate how this tool has evolved into a vital characterization methodology for analyzing lignin's contribution to reduced recalcitrance both before and after pretreatment.

At the cornerstone of this green industrial revolution is the growing realization that the conversion of biomass to biofuels, either by a biological approach or thermochemical technologies, will benefit from selectively modifying plant cell wall structures to facilitate their bioconversion.[12–14] Lignocellulosic biomass is naturally resistant to deconstruction from many microbes and enzymes, which is collectively defined as recalcitrance. Plant cell walls are complex and dynamic structures comprised primarily of cellulose, hemicellulose and lignin. Lignin is generally considered the most recalcitrant component of plant cell walls. Recent studies have clearly demonstrated that the biological conversion of biomass to biofuels benefits from reduced lignin content and alterations in its structure. Chen and Dixon produced transgenic alfalfa lines through down-regulation of *p*-coumarate 3-hydroxylase (C3H) and hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT) genes, resulting in a 35–51% reduction in lignin content.[15] These transgenic alfalfa lines demonstrated improved fermentable sugar yields when compared to wild type plants. Subsequent studies observed substantial changes in monolignol units ratio and relative abundance of inter-unit linkages in lignin.[16,17] Studies by Davison *et al.* have examined the natural variations in lignin structure for poplar and documented that both the lignin content and the syringyl (S):guaiacyl (G) ratio contributed to the ease of release of xylose from acid pretreatment.[18] Corredor *et al.* have reported that forage sorghums with a low S:G value were more readily enzymatically hydrolyzed after an acidic pretreatment.[19] Likewise, it is well appreciated that the ease of chemical pulping can be correlated with percentage of syringyl unit present in hardwoods. Huntley *et al.*, Pilate *et al.* and Lapierre *et al.* have all reported that for a series of transgenic poplars with increasing S:G ratios an increase in chemical pulping efficiency can be achieved.[20–22] Given these benefits that can be captured by controlling the plant cell wall structures and the key role which lignin plays in the utilization of plant biomass, it is clear that future research efforts will continue to develop new transgenic plants with variations in lignin content and structures. These advances will lead to a renaissance in lignin characterization, especially in genetically altered plants.

Lignin is an irregular and heterogeneous polyphenolic biopolymer in plants synthesized by radical polymerization of phenylpropanoid units (*i.e.*, monolignols), namely, coniferyl, sinapyl and *p*-coumaryl alcohols (see Fig. 1), which correspond

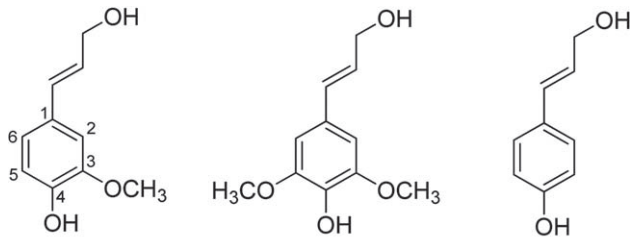
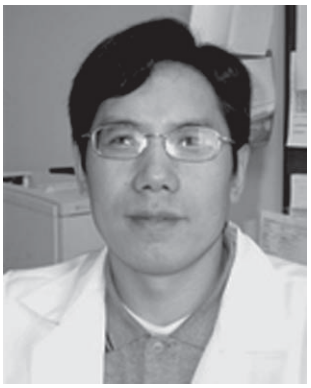


Fig. 1 Monolignols involved in lignin biosynthesis.[23–25]



Yunqiao Pu

Dr Y. Pu is a senior research scientist at Georgia Institute of Technology and his research is directed at lignin chemistry and biomass characterization. His work on lignin chemistry is focused on defining the relationship between lignin structure and the reactivity of biomass towards bioconversion processes. Whereas, his studies on cellulose are directed at providing new insight into the fundamental relationship between cellulose crystallinity and enzymatic deconstruction for biofuel production.

Shilin Cao

Shilin Cao received his PhD degree in Pulp & Papermaking Engineering from the South China University of Technology, China (2006). He is currently an associate professor at college of Material Engineering, Fujian Agriculture and Forestry University, Fuzhou, China. He has made contributions to research in bamboo pulping and bleaching with a special emphasis on oxygen delignification. At present, his main research interests are in the biomass conversions to biofuel and chemicals.

Arthur J. Ragauskas

Professor Art Ragauskas held the first Fulbright Chair in Alternative Energy and is a Fellow of the International Academy of Wood Science and TAPPI. His research program at Georgia Institute of Technology is seeking to understand and exploit innovative sustainable bioresources. This multifaceted program is targeted to develop new and improved applications for nature's premiere renewable biopolymers for biomaterials, biofuels, biopower, and bio-based chemicals. He is GA Tech's team leader for BioEnergy Science Center (BESC) research efforts and team leader for several biorefinery consortium programs.

to the guaiacyl, syringyl and *p*-hydroxyphenyl structures of lignin, respectively.[23–25] Softwood lignin is composed mainly of guaiacyl units and trace amounts of *p*-hydroxyphenyl units, while hardwood lignin generally comprises guaiacyl and syringyl units with minor amounts of *p*-hydroxyphenyl units. Grass lignin typically contains considerable amounts of all three types of monolignol units. The lignin macromolecule is primarily connected through carbon-carbon and carbon-oxygen bonds between building blocks of phenylpropane monomers, with no regular inter-unit repeating structure observed. The structural complexity of lignin provides a significant challenge to modern analytical techniques. Although the exact structure of protolignin in plant is still unknown, improvements in methods for identifying lignin degradation products and advancements in spectroscopic techniques have enabled researchers to elucidate the predominant structural features of lignin. The most common and major inter-unit linkages in lignin, such as β-*O*-4, α-*O*-4/β-5, β-β, dibenzodioxocin, β-1 and 4-*O*-5, have been identified and their relative proportions are dependant upon biomass sources as well as the lignin isolation processes employed.[23,26–28]

Analyses of lignin chemical structural characteristics including its monolignol ratio, inter-unit linkages and functional groups have advanced substantially over the last two decades, with many wet-chemistry methods such as oxidative degradation, thioacidolysis and titration being displaced and/or supplemented by spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy.[29–35] NMR is a powerful tool for detailed structural characterization of lignin macromolecule and has been substantially developed and frequently employed in elucidating lignin structures.[36–41] Lundquist *et al.*[42,43] have shown that ^{1}H NMR can be employed to quantify carboxylic acids, formyl, aromatic hydrogens, and methoxyl group in lignin. ^{13}C NMR has been extensively employed for lignin structural analysis, benefitting from a broader spectral window and enhanced signals resolution as compared to ^{1}H NMR.[37] Nonetheless, these one-dimensional (1D) NMR techniques (*i.e.*, ^{1}H and ^{13}C NMR) usually suffer from severe signal overlap issues which nowadays are addressed with a host of two-dimensional (2D) NMR correlation experiments. The use of 2D NMR has been instrumental in advancing the analysis of lignin structure, especially in determination of new lignin subunits and the presence of lignin-carbohydrate complexes.[40,44–47] However, it also has limitations since it is not quantitative and spectral overlap of lignin functionality still occurs.

A complementary approach to the limitations of the general 1D (*i.e.*, ^{1}H and ^{13}C NMR) and 2D correlation NMR approaches is to 'selectively tag' lignin functional groups with a NMR active nucleus and then analyze the derivatized lignin by NMR.[48–55] For example, several ^{19}F reagents have been developed to tag carbonyl groups and determine their concentration by ^{19}F NMR.[56–58] Likewise, Lebo *et al.*,[59] Argyropoulos *et al.*[60,61] and Zawadzki *et al.*[62] reported the use of trimethyl phosphite to detect quinonoid structures in lignin using liquid and solid state ^{31}P NMR. Argyropoulos group performed pioneering work using ^{31}P NMR to determine hydroxyl groups in lignins isolated from wood as well as derived from pulping and papermaking process in the early and mid-1990s.[63–66] The acquisition conditions of this methodology and signal assignments of lignin structure units were developed using lignin model compounds.[63–65] With suitable phosphitylation phosphorous reagents, different hydroxyl groups in lignin belonging to aliphatic, carboxylic, guaiacyl, syringyl, *p*–hydroxyphenyl units, catechols as well as guaiacyl with carbon substituents at C_5 position, can be readily quantified with ^{31}P NMR spectroscopy.[67–74] Hydroxyl groups, especially free phenoxy groups, are one of the most important functionalities affecting physical and chemical properties of lignin.[24] These functional groups exhibit a prominent role in defining reactivity of lignin to promote cleavage of inter-unit linkages and/or oxidative degradation. The traditional wet chemistry methods employed to determine hydroxyl contents in lignin typically involves time-consuming and/or laborious multi-step derivatizations. For instance, the aminolysis method for phenolic hydroxyl content measurement in lignin involves acetylation, evaporation, drying, aminolysis, and gas chromatograph analysis.[75] While ^{1}H NMR has been reported to quantify carboxylic acids, phenols in C_5 substituted units, and guaiacyl phenolic hydroxyls in underivatized lignin, the aliphatic hydroxyls can't be readily measured. Quantitative ^{13}C NMR is capable of determining aliphatic and phenolic hydroxyls, however, it requires a relatively large sample size (~80–150 mg ml^{-1} solvent) and generally extended NMR acquisition time up to ~36 h for a satisfactory signal/noise ratio. ^{31}P NMR method demonstrates a unique advantage in measurement of lignin hydroxyls in a single spectrum, providing quantitative information for various types of major hydroxyl groups in a relatively short experimental time and with small amounts of sample. Compared to ^{1}H NMR, the large range of chemical shifts reported for the ^{31}P nucleus generates a better separation and resolution of signals. In addition, the 100% natural abundance of the ^{31}P and its high sensitivity renders ^{31}P NMR a rapid analytical tool in comparison with ^{13}C NMR.

^{31}P NMR analysis to quantitatively determine hydroxyl groups in lignin has been widely applied to lignins isolated from industrial process streams such as pulping and bleaching. Given the importance and need for facile direct analysis of the fundamental structure of lignin and its conversion chemistry in biomass to biofuels, the ^{31}P NMR technique has begun to see growing application in biomass chemistry, lignin-related biofuel, and biofuel precursors research. This review summarizes the recent results of ^{31}P NMR analysis for lignins isolated from native and transgenic plants including softwoods, hardwoods and grasses, as well as from biomass after various pretreatments in conversion of biomass to biofuel process. The application of this methodology to provide a rapid analytical tool for pyrolysis bio-oils from thermal deconstruction of lignin/biomass and biodiesel precursors is also discussed.

2. The basics of ^{31}P NMR analysis of lignin

^{31}P NMR method has been applied in various substrates including coal, coal-derived products, and biomass lignins, involving phosphitylation of hydroxyl groups in a substrate using a ^{31}P reagent followed by quantitative ^{31}P NMR analysis.[76–78] For lignin and lignin derived products, the most common phosphitylating reagent employed is 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP). TMDP reacts with hydroxyl groups in lignin arising from aliphatic, phenolic, and carboxylic acids groups in the presence of an organic base,

such as pyridine, to give phosphitylated products (Fig. 2).[55,70] Pyridine is selected as the base in the solvent mixture to capture the liberated hydrogen chloride and drive the slightly exothermic overall phosphitylation reaction to total conversion.[77] The phosphitylated hydroxyls can then be quantitatively assessed against an internal standard that demonstrates adequate stability and satisfactory resolution from lignin hydroxyl regions in a ^{31}P NMR spectrum. A typical ^{31}P NMR spectrum of a switchgrass ball-milled lignin derivatized with TMDP using cyclohexanol as internal standard is illustrated in Fig. 3.

^{31}P NMR analysis typically requires the difference among chemical shifts of derivatized groups be sufficiently broad to permit identification and integration. Trivalent and pentavalent phosphorous reagents have been examined and the largest chemical shift differences were observed with trivalent phosphorous reagents.[79,80] Wroblewski *et al.*[78] examined five trivalent ^{31}P reagents to derivatize organic model compounds including phenols, aliphatic alcohols, aromatic acids, aliphatic acids, amines, and thiols. The results showed that 2–chloro–1,3,2–dioxaphospholane (CDP), 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP), and 2–chloro–4,4,5,5–tetraethyl–1,3,2–dioxaphospholane (TEDP) served as suitable reagents for identification of various OH groups in liquid coal-derived samples, with TMDP providing the best results. 2–chloro–1,3,2–dioxaphospholane (CDP) was reported to allow quantifying carboxylic and guaiacyl phenolic hydroxyls in lignin as well as differentiating primary and secondary OH groups between *erythro*– and *threo*–conformations of β-*O*-4 structures.[68] However, signal overlap was observed between syringyl phenolic and C_5 substituted guaiacyl phenolics when using this reagent, and it has not been frequently applied to native lignin, especially hardwood lignin. Compared to CDP, TMDP provides better signal resolution of various phenolic moieties as well as better stability of the phosphitylated products.[81]

Another advantage of the TMDP/^{31}P NMR technique is that it is well developed and a database of model compounds spectral information is available.[82–84] The quantitative information gained from this technique has been verified against other

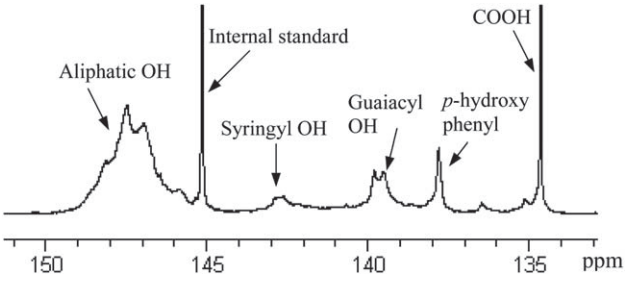


Fig. 3 Quantitative ^{31}P NMR spectrum of a switchgrass ball-milled lignin derivatized with TMDP using cyclohexanol as internal standard.

techniques such as benzyl acetate/GC, ^{1}H–NMR, ^{13}C–NMR, FT-IR and wet chemistry methods during an international round robin lignin study.[85–87] For example, Granata *et al.* studied a series of lignin samples using TMDP/^{31}P NMR and observed that the measured phenolics content was in good agreement with other analytical results.[81] Jiang *et al.* applied the Mannich reaction to a variety of lignin model compounds and documented that ^{31}P NMR analysis allowed quantification of various aromatic groups bearing free phenolic hydroxyls, including *p*-hydroxyphenyls, catechols, guaiacyl units and phenols with carbon substituents at C_5 or C_6 positions.[88] A comprehensive compilation of hydroxyl groups in lignin and their typical chemical shifts/integration ranges using TMDP/^{31}P NMR analysis is summarized in Table 1.[81,85,89–91]

Although cyclohexanol is frequently used as an internal reference in phosphitylation/^{31}P NMR analysis of lignin, Zawadzki and Ragauskas examined a variety of N-hydroxy compounds as internal standards for lignin analysis including N–hydroxy–phthalimide, 1–hydroxy–7–azabenzotriazole, N–hydroxy–5–norborene–2,3–dicarboximide, and N–hydroxy–1,8–naphthalimide.[92] The results showed that these compounds were suitable as an internal standard for ^{31}P NMR analysis of lignin with chemical shifts of phosphitylated N-hydroxy compounds (*i.e.* δ 150.7–153.6 ppm) well separated from lignin-derived components. A quantitative ^{31}P NMR spectrum of a TMDP

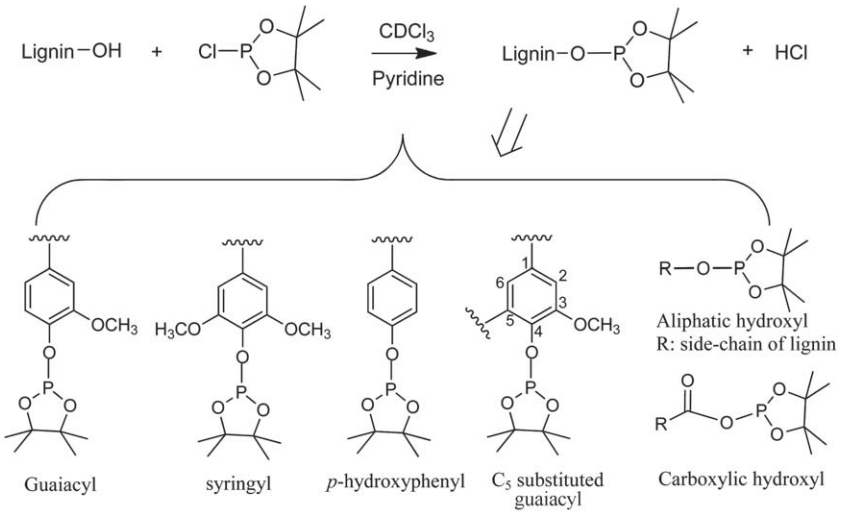


Fig. 2 Phosphitylation of hydroxyl groups in lignin structural units with 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP).

Table 1 Typical chemical shifts and integration regions for lignins in a ^{31}P NMR spectrum.[81,85,89–91]

Structure	δ (ppm)
(1) Aliphatic OH	145.4–150.0
(2) Phenols	137.6–144.0
(2a) C_5 substituted	140.0–144.5
β–5	~143.5
Syringyl	~142.7
4–O–5	~142.3
5–5	~141.2
(2b) Guaiacyl	139.0–140.2
(2c) Catechol	~138.9
(2d) *p*–hydroxyphenyl	~137.8
(3) Carboxylic acid OH	133.6–136.0

treated softwood lignin using N-hydroxy-5-norbornene-2,3-dicarboximide as an internal standard is shown in Fig. 4.

3. Experimental considerations

For ^{31}P NMR analysis of lignin, the solvent employed is usually a mixture of anhydrous pyridine and deuterated chloroform (~1.6 : 1.0, v/v) containing a relaxation agent (*i.e.*, chromium(III) acetylacetonate) and an internal standard. Pyridine is used as the base to capture the hydrogen chloride liberated during phosphitylation reaction and should be in excess relative to the phosphorus reagent since the excess HCl released during derivatization is capable of inducing decomposition of the derivatized compounds. Deuterated chloroform provides a deuterium signal for locking in NMR experiments, dissolves the derivatized lignin sample as well as prevents precipitation of the pyridine-HCl salt. For select lignin and bio-oil/biodiesel samples with limited solubility, a third solvent *N*,*N*-dimethylformamide, is usually introduced in the solvent system to help dissolve samples and prevent precipitation of derivatized products.

The lignin phosphitylation reaction requires lignin samples to be dried which is usually accomplished in a vacuum oven ~35 °C for overnight. An accurately weighed lignin sample (~20 mg) is dissolved in a NMR solvent mixture (0.50 ml). TMDP reagent (~0.05–0.10 ml) is added and stirred for a short period of time at room temperature. The reaction mixture is then transferred into a 5 mm NMR tube for ^{31}P NMR analysis. Since the derivatization reagent is moisture sensitive, all efforts need to be directed at reducing exposure to water.

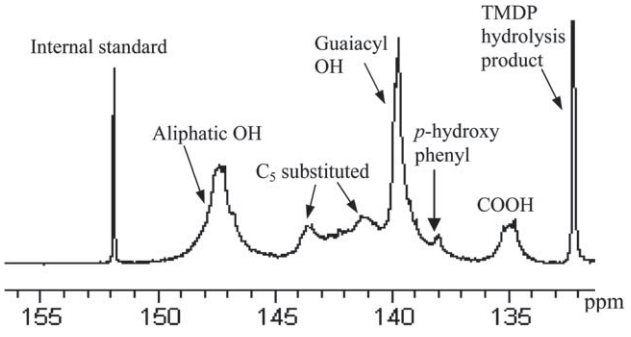


Fig. 4 Quantitative ^{31}P NMR spectrum of a softwood lignin derivatized with TMDP using N-hydroxy-5-norbornene-2,3-dicarboximide as internal standard.

Quantitative ^{31}P NMR spectra are generally recorded with a long pulse delay which is at least 5 times greater than the longest spin–lattice relaxation time (T_1) of ^{31}P nucleus to ensure phosphorus nuclei to reach thermal equilibrium prior to a subsequent pulse. Chromium(III) acetylacetonate in solvent system is used as relaxation agent to shorten the spin–lattice relaxation time of phosphorus nuclei.[90–93] Typically, a 25-s pulse delay is considered appropriate for quantitative ^{31}P NMR analysis of lignin. In addition, an inverse gated decoupling pulse is employed to eliminate the nuclear Overhauser effects for quantitative purpose. Using a 90° pulse and the conditions above, 128–256 acquisitions (~1– 2 h) at room temperature are sufficient to acquire a spectrum. Chemical shifts are usually calibrated relative to the phosphitylation product of TMDP with water (sample moisture), which gives a sharp and stable signal at 132.2 ppm in pyridine-$CDCl_3$ solvent.

4. Applications

4.1 ^{31}P NMR analysis of native plants lignin

Table 2 summarizes the results of quantitative ^{31}P NMR analysis of TMDP phosphitylated lignin samples isolated from various biomass sources including softwoods, hardwoods and grasses.[94–104] Among the various hydroxyl groups, the aliphatic hydroxyl signal is typically the dominant in lignin. Usually, a minor amount of carboxylic OH groups (*i.e.* 0.02–0.29 mmol g^{-1}) is observed in lignin from native woody biomass and grasses. The ^{31}P NMR data (Table 2) showed that lignin isolated from softwood (*i.e.*, pine and black spruce) had the following order of hydroxyl contents: aliphatic OH > phenolic OH > carboxylic OH. The major phenolic hydroxyls in native softwood lignin appeared to be guaiacyl phenolics with a minor amount of *p*-hydroxyphenyl, which is consistent with the overall G/H composition of softwood lignin. While syringyl phenolics are usually not detected in natural softwood lignin, C_5 substituted guaiacyl phenolics have been observed in softwood lignin using ^{31}P NMR. For example, Sannigrahi *et al.* employed ^{31}P NMR to characterize ball-milled lignin from loblolly pine and determined 0.08 mmol g^{-1} of C_5 substituted phenolic hydroxyl groups.[94] Guerra *et al.* reported that lignin isolated from southern pine contained 0.43–0.50 mmol g^{-1} of phenols in C_5 substituted units based on different isolation methods.[95] Milled-wood lignin (MWL) isolated from black spruce was found to have 0.36–0.50 mmol g^{-1} C_5 substituted phenolic OH groups (Table 2).[95,97,98] Compared to enzymatic mild acidolysis lignin (EMAL) isolated from normal wood of southern pine, lignin in compression wood had a lower content of guaiacyl phenolic OH, higher content of *p*-hydroxyphenyl and a similar content of carboxylic OH group.[95]

Hardwood lignin typically contains guaiacyl and syringyl units and its hydroxyl groups can be readily determined using ^{31}P NMR analysis after phosphitylation with TMDP. Table 2 demonstrates a general order of hydroxyl group content in hardwood lignin as following: aliphatic > guaiacyl phenolic ~ syringyl phenolic > *p*-hydroxyphenyl ~ carboxylic OH. Hallac *et al.* reported the primary phenolic hydroxyls in MWL lignin of *Buddleja davidii* were from guaiacyl unit and no *p*-hydroxyphenyls was detected.[35]

Table 2 Hydroxyl group contents of lignin isolated from native biomass as determined by ^{31}P NMR analysis

		Phenolic OH, mmol g^{-1}				
	Aliphatic OH mmol g^{-1}	C_5 substituted	Syringyl	Guaiacyl	*p*-hydroxy phenyl	COOH mmol g^{-1}
Loblolly pine, MWL[94]	4.16	0.08		0.57	0.12	0.02
Southern pine[95]						
Normal wood, CEL		~0.46				~0.11
Normal wood, MWL		~0.50				~0.16
Normal wood, EMAL		0.43		0.79	0.12	0.11
Compression wood, EMAL		0.48		0.57	0.38	0.10
Black spruce						
MWL[95]	4.27	0.36		0.77		0.21
MWL[97]	4.21	0.50		0.76	0.08	0.15
MWL[98]	4.13	0.44		0.67	0.09	0.11
EAL[98]	4.92	0.30		0.72	0.06	0.09
Norway spruce[96]						
MWL	1.03[a]	0.06		0.14	0.01	0.02
EMAL	1.25	0.08		0.17	0.01	0.03
CEL	0.92	0.08		0.12	0.02	0.02
P. tremuloides						
MWL[98]	4.53	0.29	0.23	0.37	0.17	0.14
EAL[98]	3.91	0.22	0.24	0.33	0.14	0.11
MWL[99]	5.72	0.13	0.16	0.25	0.20	0.06
Douglas fir, EMAL[97]		0.41		0.84	0.10	0.13
White fir, EMAL[97]		0.56		0.93	0.11	0.19
Redwood, EMAL[97]		0.63		1.06	0.16	0.16
E. globules, EMAL[97]			0.62	0.35	0.02	0.15
E. ulmoides oliv, MWL[100]	4.05		0.19	0.19	0.03	
B. davidii, MWL[35]	4.51	0.27		0.43		0.03
Wheat straw[101]						
MWL	3.49	0.18	0.09	0.51	0.68	0.12
Dioxane acidolysis	3.80	0.13	0.10	0.51	0.50	0.18
Micanthus × giganteus						
MWL[102]	5.54		0.14	0.38	0.32	0.18
MWL[103]	4.00		0.22	0.67	0.64	0.13
Switchgrass, MWL[104]	3.88	0.20		0.48	0.32	0.29

[a] expressed as moles/C_9; MWL: milled wood lignin; EMAL: enzymatic mild acidolysis lignin; CEL: cellulolytic enzymatic lignin; EAL: enzymatic/acidolysis lignin.

Akim *et al.* demonstrated that MWL lignin from a two-year old poplar had a *p*-hydroxyphenyl content of 0.20 mmol g^{-1} and syringyl phenolic OH content of 0.16 mmol/g.[99] Compared to softwood and hardwood lignin, grass lignin was found to have a much higher *p*-hydroxyphenyl content (Table 2). Crestini *et al.* reported 0.68 mmol g^{-1} of *p*-hydroxyphenyl group in wheat straw ball-milled lignin and El Hage *et al.* observed 0.64 mmol g^{-1} in Miscanthus ball-milled lignin.[101,103] It appeared that the free phenolic hydroxyls in grass were primarily from *p*-hydroxyphenyl and guaiacyl units (Table 2).

In combination with a degradative method termed Derivatization Followed by Reductive Cleavage (DFRC), quantitative TMDP/^{31}P NMR analysis has also been used to determine β-aryl ether linkages linked to condensed and noncondensed aromatic moieties in lignin, including dibenzodioxocins.[97] Guerra *et al.* employed ^{31}P NMR analysis combined with DFRC to analyze EMAL lignin isolated from softwood and hardwood and demonstrated that the total number of β-aryl ether structures in EMAL lignin determined by DFRC/^{31}P NMR analysis was in agreement with the results measured by thioacidolysis.[105] The total uncondensed β-*O*-aryl linkage in EMAL lignin of spruce was reported to be 770 μmol g^{-1} by DFRC/TMDP/^{31}P NMR analysis and 766 μmol g^{-1} by thioacidolysis, respectively. A comparable analysis of EMAL lignin from white fir yielded a ~900 μmol g^{-1} of uncondensed β-*O*-aryl linkage, which is consistent with our current understanding of the structure of softwood and hardwood lignin.

4.2 Transgenic plants lignin

Genetic modification to reduce the native recalcitrance of biomass is a promising route to yield the next generation of plants with enhanced biofuel production potential.[12,16,106] One of the most promising approaches to reduce recalcitrance in plants involves genetic manipulation of enzymes that catalyze synthesis of lignin precursors in lignin biosynthesis pathways, resulting in lignin content and/or structure alterations. Using ^{31}P NMR methodology, Akim *et al.* investigated structural features of ball-milled lignins isolated from a control wild type, a cinnamyl alcohol dehydrogenase (CAD) down-regulated line, and a caffeic acid/5-hydroxyferulic acid O-methyl transferase (COMT) down-regulated transgenic poplar.[99] According to the ^{31}P NMR results (Table 3), Akim *et al.* documented that moderate CAD down-regulation (70% deficient) resulted in no drastic changes in structures of poplar lignin. More severe CAD depletion on 6-month old poplar led to a slight increase in the amount of phenols in C_5 substituted units which the authors suggested was indicative of a higher degree of cross-linked lignin. Compared to

Table 3 Hydroxyl group content in lignin isolated from control and transgenic poplar[99,a]

		Phenolic OH, mmol g^{-1}				
	Aliphatic OH mmol g^{-1}	C_5 substituted	Syringyl	Guaiacyl	*p*-hydroxy phenyl	COOH mmol g^{-1}
2-year old trees						
Control	5.72	0.13	0.16	0.25	0.20	0.06
70% CAD deficient	5.23	0.16	0.17	0.25	0.21	0.07
90% COMT deficient	5.30	0.15	0.09	0.36	0.20	0.06
COMT/CAD deficient	5.06	0.20	0.08	0.41	0.11	0.08
6-month old trees						
Control		0.14	0.17	0.23	0.17	—
90% CAD deficient		0.21	0.18	0.24	0.14	—

[a] CAD: cinnamyl alcohol dehydrogenase; COMT: caffeic acid/5-hydroxyferulic acid O-methyl transferase.

wild type control, COMT down-regulation (90% deficient) yielded a poplar lignin with a lower content of syringyl and aliphatic OH group as well as an increased guaiacyl phenolic OH amount (Table 3). The *p*-hydroxyphenyl and carboxylic OH content was observed to remain unchanged after COMT down-regulation.[99]

4.3 ^{31}P NMR analysis of lignin from biomass pretreatment processes

To facilitate enzymatic saccharification of cellulose, biomass typically requires pretreatment which usually involves elevated temperatures (*i.e.*, ~150–220 °C) using either acidic or alkaline processing conditions, such as organosolv, steam explosion, and dilute acid pretreatment.[107–109] To-date, effective utilization of lignocellulosic biomass for biofuels by the biological approach is generally predicated on pretreatment technologies that can reduce biomass recalcitrance and yield cellulose and hemicelluloses more amenable to hydrolytic enzymes.[110] The natural recalcitrance of biomass is attributed, in part, to the complex structural characteristics of lignin present in plant cell walls. Understanding the effects of pretreatments on chemical features of biomass lignin provides valuable insights for improving pretreatment technologies. Table 4 summarizes results of quantitative ^{31}P NMR analysis of biomass lignin isolated from varying pretreatment processes.[34,39,81,94,104,111]

When compared to the control biomass lignin, a two-stage acid pretreatment led to a decreased aliphatic and an increased phenolic hydroxyl content in lignin revealed by ^{31}P NMR analysis.[94] Sannigrahi *et al.* reported that ethanol organosolv pretreatment (EOP) of loblolly pine resulted in an ethanol organosolv lignin (EOL) with a higher content of guaiacyl phenolic hydroxyl, *p*-hydroxyphenyl and carboxylic hydroxyl groups.[94,111] Hallac *et al.* applied ^{31}P NMR to determine chemical transformations of *Buddleja* davidii lignin during ethanol organosolv pretreatments with various pretreatment severities.[39] Compared to the milled wood lignin from unpretreated *B.* davidii, the amount of phenolic OH in both C_5 substituted and guaiacyl units increased significantly in EOLs after ethanol organosolv pretreatment, which was in agreement with ^{13}C NMR data. The aliphatic OH groups in *B.* davidii EOLs were observed to decrease in content by 41–59% and this decrease of aliphatic OH was enhanced as the pretreatment severity increased. Coupled with ^{13}C NMR and HSQC analysis, Hallac *et al.* suggested that the loss of aliphatic hydroxyl groups during EOP was attributed to the loss of γ-methylol group as formaldehyde and OH groups on side chain to form β-1 linkages.[39]

El Hage *et al.*[34] pretreated Miscanthus using ethanol organosolv pretreatment at differing pretreatment severities and investigated the effects of EOP on Miscanthus lignin structural features. ^{31}P NMR analysis showed that EOP resulted in a decrease of aliphatic hydroxyl content and an increase in phenolic hydroxyl groups in Miscanthus EOLs.[34,103] An increase in severity of the EOP enhanced the decrease of aliphatic hydroxyl groups and increased the total phenolic OH content in Miscanthus EOLs. Based on the ^{31}P NMR results together with ^{13}C NMR and FT-IR analysis, El Hage *et al.*[34] proposed that EOP resulted in extensive aryl-ether bond hydrolysis of Miscanthus lignin and that cleavage of α–aryl ether bonds was the primary reaction responsible for lignin depolymerization. Using ^{31}P NMR analysis, Samuel *et al.*[104] documented that dilute acid pretreatment led to a 27% decrease in aliphatic hydroxyl content and a 25% increase in phenolic hydroxyl content in switchgrass lignin, while the OH content in *p*-hydroxy phenyl and carboxyl remained relatively unchanged. The observed differences in lignin structure upon various pretreatment are undoubtedly due to the variations in structure of the starting biomass as well as the pretreatment conditions.

4.4 Lignin/biomass bio-oils

Regardless of the bioprocessing technology employed, all the current biological processing platforms for conversion of plant polysaccharides to biofuels will result in a large quantity of lignin as a byproduct.[112] Although a certain amount of this lignin (~30–50%) can be utilized as an energy resource for thermal requirements of a modern biological-based cellulosic ethanol plant, the excess is frequently used for combustion to generate green biopower.[113] Conversion of this under-utilized lignin stream into a liquid biofuel precursor, in particular a pyrolysis oil by thermal depolymerization, is receiving increased interest.[114] The high oxygen content in bio-oils obtained from pyrolyzing biomass has been reported to present problems in their applicability, including high viscosity values, low heating values, corrosiveness and a tendency to polymerize. Many of these properties are due, in part, to the presence of alcohols, phenols and carboxylic acids.[115–117] The presence of these groups also makes it difficult to blend bio-oils with conventional fossil fuels.[118] Phosphitylation of a bio-oil followed by ^{31}P NMR analysis can provide a rapid

Table 4 ^{31}P NMR analysis of lignin isolated from pretreated biomass

		Phenolic OH, mmol g^{-1}				
	Aliphatic OH mmol g^{-1}	C_5 substituted	Syringyl	Guaiacyl	*p*-hydroxy phenyl	COOH mmol g^{-1}
Loblolly pine						
Acid pretreated[94]	3.42	0.34		1.82	0.06	
EOP pretreated[111]	4.70	1.80		1.20	0.10	
EOL[111]	7.30	0.60		1.40	0.40	0.30
Aspen[a]						
steam explosion[81]	0.67		0.23	0.13		0.06
Yellow poplar[a]						
steam explosion[81]	0.53		0.34	0.18		0.08
Mixed hardwood[a]						
Alcell organosolv[81]	0.33		0.49	0.26		0.06
Buddleja davidii[39]						
EOL1	2.67	0.98		1.66		0.16
EOL2	2.51	0.98		1.53		0.17
EOL3	1.86	1.07		1.66		0.15
Miscanthus × giganteus[34]						
EOL1 (CS 1.75)	3.11	0.72		0.91	0.49	0.22
EOL2 (CS 2.08)	1.78	0.90		1.15	0.51	0.16
EOL3 (CS 2.38)	1.55	0.87		1.16	0.51	0.17
EOL4 (CS 2.93)	1.26	1.70		1.58	0.65	0.28
Switchgrass[104]						
dilute acid pretreated	2.83	0.35		0.57	0.33	0.33

[a] expressed as moles/C_9; EOL: ethanol organosolv lignin; CS: combined severity.

tool for monitoring the hydroxyl-based functional groups present in biomass/lignin pyrolysis oils.

Gellerstedt *et al.* used ^{31}P NMR to analyze two lignin bio-oils obtained from pyrolysis of commercial spruce sodium lignosulfonate and steam-exploded birch wood lignin.[119] Lignin pyrolysis was performed in a direct one-step manner in which formic acid and alcohol mixtures were used as the reaction medium at reaction temperature of 300 °C.[119,120] The resulting bio-oils were observed to contain a substantial number of carboxyl groups as well as phenolic compounds with several distinctly different substitution patterns as revealed by ^{31}P NMR analysis (Table 5). Only a minor portion of aliphatic hydroxyl groups remained in lignin pyrolysis bio-oils in agreement with other analytical data.[119] Fu *et al.* employed ^{31}P NMR method to characterize organic components of a pyrolysis oil produced from chromated copper arsenate (CCA) treated southern pine wood by pyrolysis over the temperature range of 275–350 °C.[121,122] As the pyrolysis temperature increased, both untreated and CCA-treated wood had an increase in the amount of aliphatic alcohols, total phenols, and carboxylic acids in pyrolysis products (Table 6). ^{31}P NMR analysis showed that a greater amount of non-condensed phenolic units than C_5 substituted phenolic units were observed in the pyrolysis oils and Fu *et al.* suggested that most lignin degradation products in the pyrolysis products were monomeric phenols resulted from cleavage of β-aryl ether linkages during pyrolysis.

Recently, David *et al.* pyrolyzed assorted woody biomass resources and used ^{31}P NMR to determine chemical nature of the hydroxyls present in the produced bio-oils as summarized in Table 7.[123] The pyrolysis reaction was accomplished in a micro-reactor with a 2-min residence time at 400 °C. The ^{31}P NMR results showed that the bio-oils obtained from sweet gum had a lower total hydroxyl content (1.54 mmol g^{-1}) than from loblolly pine (2.62 mmol g^{-1}). The total phenolic OH content of bio-oil derived from loblolly pine milled-wood lignin was greater than that obtained from starting loblolly pine biomass, while the aliphatic OH content in bio-oil loblolly pine MWL was lower. No phenolic and carboxylic OH groups were observed in pyrolysis product of loblolly pine cellulose. ^{31}P NMR was also utilized by David *et al.* to determine water content in bio-oils produced from loblolly pine using TMDP as the phosphitylating reagent.[123] This ^{31}P NMR methodology for measuring water content in pyrolysis oils was based on earlier studies by Hatzakis *et al.* which used ^{31}P NMR spectroscopy to determine water content in olive oil.[124] For pyrolysis oil produced from loblolly pine, the water content measured by ^{31}P NMR was reported to be in good agreement with the well-established Karl Fischer titration methodology.[123]

Table 5 Hydroxyl group contents in bio-oils from lignin pyrolyzed using formic acid/ethanol[119,a]

		Phenolic OH, mmol g^{-1}			
Bio-oils	Aliphatic OH mmol g^{-1}	144–142 ppm	140–139 ppm	139–138 ppm	COOH mmol g^{-1}
MK 79	0.07	0.55	0.02	0.65	0.93
MK 84	0.40	1.54	0.09	0.12	0.42

[a] MK 79: from spruce sodium lignosulfonate; MK 84: from steam-exploded birch wood lignin.

Table 6 Hydroxyls in pyrolysis products from untreated and CCA-treated southern pine wood[121,122,a]

Pyrolysis oils	Aliphatic OH mmol g^{-1}	Phenolic OH, mmol g^{-1}		COOH mmol g^{-1}
		C_5 substituted	noncondensed	
At 300 °C				
untreated	6.36	0.86	1.67	0.45
CCA treated	7.97	0.77	1.28	0.45
At 325 °C				
untreated	6.75	0.98	1.63	0.46
CCA treated	8.53	0.75	1.35	0.49
At 350 °C				
untreated	7.08	0.94	1.67	0.64
CCA treated	8.53	0.90	1.32	0.54

[a] CCA: chromated copper arsenate treatment.

4.5 ^{31}P NMR analysis in lignin hydrogenolysis

Nagy *et al.* examined the potential of catalytic hydrogenolysis/hydrogenation of ethanol organosolv lignin to convert lignin from a low grade fuel to potential biofuel precursors or value-added chemicals.[125] Hydrogenolytic cleavage of an inter-unit linkage in lignin increased hydroxyl group contents of final products, while hydrogenation of the aromatic ring decreased phenol concentrations and elevated contents of aliphatic hydroxyl groups. TMDP phosphitylation followed by ^{31}P NMR analysis was found to provide a reliable means of monitoring hydroxyl group changes in lignin after catalytic hydrogenolysis/hydrogenation. A series of homogeneous catalysts were examined on ethanol organosolv lignin and results of ^{31}P NMR analysis were summarized in Table 8.[125] Catalyst $NaBH_4/I_2$ resulted in a decreased phenolic OH content and an increased carboxyl OH groups in EOL lignin, in agreement with the ^{1}H NMR results. The total OH content (*i.e.* phenolic and aliphatic) in EOL lignin were observed to increase after treatment with RANEY®-Ni and Pt/C as a catalyst and Nagy *et al.* proposed that hydrogenolytic cleavage of aryl-*O*-aryl and aryl-*O*-aliphatic linkages in lignin occurred.

4.6 ^{31}P NMR analysis in biodiesels

Although bioethanol represents the predominant 1st and 2nd generation biofuel, biodiesel from plant oils, fats and in the future algae continues to garner regional support as it has several attractive attributes, such as ease of incorporation into existing fuels distribution systems, ready utilization in modern diesel combustion engines, and favourable emission profiles.[126] Currently, the most widely-used method to produce biodiesel is catalytic transterification of vegetable oils or animal fats with an alcohol. This process also yields glycerol, fatty acids and partially substituted glycerol as a by-product.[127–129] To achieve a high conversion yield and a low contaminant level, it is essential to monitor chemical structures of the incoming feedstock and reaction products. The currently used analytical methods are primarily chromatographic, including: high pressure liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS), near infrared spectroscopy (NIRS), and wet chemical techniques (potentiometric, iodometric titration), which are often time consuming due to sample preparation, extended analysis time and/or complicated data analysis.

Recently, Nagy *et al.* developed a TMDP/^{31}P NMR methodology to characterize biodiesel process streams that include mixtures of alcohols, fatty acids, glycerol, mono- and/or di-substituted glycerides.[129–132] A database of ^{31}P NMR chemical shift information of the relevant biodiesel precursors was established with analytical compounds (Table 9).[129,132] A typical ^{31}P NMR spectrum of partially processed biodiesel oil from commercial biodiesel operations is shown in Fig. 5. Subsequently, this methodology was employed to analyze a series of commercial biodiesel samples and the results were found to be comparable with the data acquired using conventional GC analysis.[129] The quantitative accuracy of the TMDP/^{31}P NMR technique was measured to within 95+% and the low detection limit was shown to be 1.9 μmol/ml. The TMDP/^{31}P NMR method can be used to measure the concentration of alcohol, free glycerol, and partially hydrolyzed triglycerides free fatty acids in a short time, thus providing a valuable research tool in biodiesel characterization. Using TMDP/^{31}P NMR, Nagy *et al.* characterized two series of biodiesel samples from industrial process stream which utilized pure and waste vegetable oil as feedstocks respectively, and reported that different feedstock resulted in final biodiesel products with different compositions.[132]

Table 7 Hydroxyls in bio-oils as determined by ^{31}P NMR[123]

Bio-oils source	Aliphatic OH mmol g^{-1}	Phenolic OH, mmol g^{-1}		COOH mmol g^{-1}
		C_5 substituted	Guaiacyl/*p*-hydroxyphenyl	
Loblolly pine	0.73	0.29	1.36	0.24
Sweet gum	0.23	0.20	1.02	0.09
Milled wood lignin of loblolly pine	0.10	0.23	2.31	0.26
Cellulose of loblolly pine	2.95	—	—	—

Table 8 Hydroxyl content in EOLs after hydrogenation with various catalysts as determined by ^{31}P NMR[125]

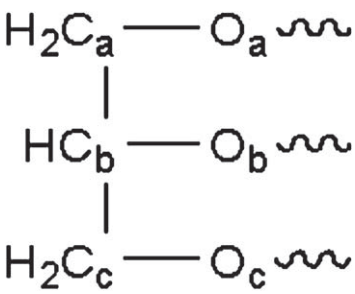
	OH content of selected groups, μmol mg^{-1}		
Catalyst	Aliphatic OH (149.0–145.6 ppm)	Total phenolic (144.4–137.6 ppm)	COOH (136.0–133.8 ppm)
Starting EOL	1.00[a]	1.05	0.00
Blank[b]	0.87	1.12	0.00
RANEY®-Ni	1.21	1.12	0.00
Pt/C	1.01	1.14	0.00
$NaBH_4/I_2$	1.08	0.81	0.38

[a] EOL aliphatic OH value is used as reference for hydroxyl content of selected groups. [b] no catalyst.

5. Conclusions and future perspective

The ^{31}P NMR analysis methodology presented in this review offers a facile and rapid tool for analysis of lignin in native and transgenic plants as well as biomass pretreatment process. This technique provides a reliable quantitative assessment of various hydroxyl groups present in lignin in a relatively short time, including guaiacyl, syringyl, C_5 substituted guaiacyl phenolics, catechols, *p*–hydroxyphenyls, aliphatic and carboxylic hydroxyls. In addition, ^{31}P NMR has also been employed to characterize lignin/biomass derived bio-oils as well as biodiesel precursors which can be used to optimize processing conditions to upgrade and improve the quality of bio-oil and biodiesel products. This method can have broad applicability for researchers involved in biomass conversion to second and third generation biofuels and its usage will predictably grow in the future.

Table 9 Chemical shifts of biodiesel precursors in ^{31}P NMR after phosphitylation with TMDP[129,132]



	Designated phosphitylated Position	δ ^{31}P NMR (ppm)[a]
Glycerol derivatives		
Mono-substituted		
1-Monopalmitoleoyl-rac-glycerol	*O*-b	146.2
	O-c	147.6
1-Octanoyl-rac-glycerol	*O*-b	146.2
	O-c	147.4
2-Oleoylglycerol	*O*-a & O-c	147.8
Di-substituted		
1,2- Dioleoylglycerol	*O*-c	147.7
1,3- Dioleoylglycerol	*O*-b	146.4
Tri-substituted/transesterified		
Glyceryl trioleate	———	———
Fatty acids		
Saturated		
Hexanoic acid	*O*-a	134.3
Palmitic acid	*O*-a	134.4
Stearic acid	*O*-a	134.4
Unsaturated		
Oleic acid	*O*-a	134.4
Linoleic acid	*O*-a	134.3
Linolenic acid	*O*-a	134.3
Biodiesel production by-products		
Free Glycerol	*O*-a & O-c	147.1
	O-b	146.1
Methanol		147.9
Ethanol		146.3
Isopropanol		146.4

[a] referenced to internal standard of cyclohexanol at *δ* 144.9 ppm.

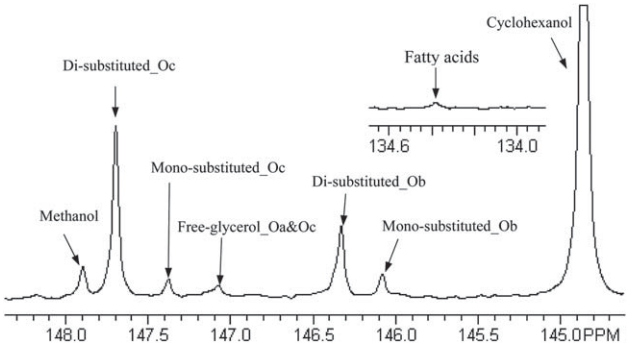


Fig. 5 Quantitative ^{31}P NMR spectrum of partially processed biodiesel oil.

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